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The non-coding *Air* RNA is required for silencing autosomal imprinted genes

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In genomic imprinting, one of the two parental alleles of an autosomal gene is silenced epigenetically by a *cis*-acting mechanism[1,2]. A bidirectional silencer for a 400-kilobase region that contains three imprinted, maternally expressed protein-coding genes (*Igf2r/Slc22a2/Slc22a3*) has been shown by targeted deletion to be located in a sequence of 3.7 kilobases[3–5], which also contains the promoter for the imprinted, paternally expressed non-coding *Air* RNA[6]. Expression of *Air* is correlated with repression of all three genes on the paternal allele[5]; however, *Air* RNA overlaps just one of these genes in an antisense orientation[6]. Here we show, by inserting a polyadenylation signal that truncates 96% of the RNA transcript, that *Air* RNA is required for silencing. The truncated *Air* allele maintains imprinted expression and methylation of the *Air* promoter, but shows complete loss of silencing of the *Igf2r/Slc22a2/Slc22a3* gene cluster on the paternal chromosome. Our results indicate that non-coding RNAs have an active role in genomic imprinting.

Most mammalian imprinted genes are found in clusters that also contain imprinted non-coding RNAs[2,7]. In many cases, expression of the non-coding RNA from one parental allele correlates with repression of linked protein-coding genes on the same allele, raising the possibility that non-coding RNAs either are required for silencing or are a consequence of the silencing mechanism. Studies of the *Igf2/Ins2/H19* imprinted gene cluster on mouse chromosome 7 have shown, however, that the *H19* non-coding RNA does not have a role in silencing the *Igf2/Ins2* protein-coding genes[8,9]. Instead, imprinted expression of *Igf2/Ins2* occurs through a silencer element[10] and an insulator element (called *H19*-DMR) close to the *H19* promoter, and imprinted expression of *H19* is thought to occur as a consequence of paternal-specific methylation of the insulator element[11,12]. Thus, despite the prevalence of imprinted autosomal non-coding RNAs, none has been shown to be required for the silencing of flanking imprinted protein-coding genes.

We have studied the paternal-specific *Air* non-coding RNA on mouse chromosome 17 that is expressed from a promoter located in intron 2 of the *Igf2r* gene. This non-coding RNA is 108 kilobases (kb), polyadenylated but apparently unspliced, and overlaps the *Igf2r* promoter[3,6]. Deletion of a 3.7-kb imprint control element (ICE) that contains the *Air* promoter releases paternal-specific silencing of three genes, *Slc22a2* and *Slc22a3* that are located upstream, and *Igf2r* that is located downstream[4,5]. Thus, the 3.7-kb ICE has a bidirectional action despite the fact that *Air* overlaps only one of these genes (ref. 5; and Fig. 1a).

To test whether imprinted expression of the *Air* RNA is required for silencing, we truncated *Air* to 4% of its length by inserting a 1.2-kb polyadenylation cassette from the β-globin gene at the downstream border of the 3.7-kb ICE to yield the *Air-T* allele (Fig. 1). The modification was designed to truncate *Air* without disrupting the function of the 3.7-kb ICE, as assessed by its ability to attract a maternal-specific methylation imprint and to show paternal-specific expression of the *Air* promoter[3,6]. Mice with a maternally inherited *Air-T* allele (*Air-T/+*, note that the maternal allele precedes the paternal one in all genotypes shown here) were identical to wild type, whereas mice with a paternally inherited *Air-T* allele (*+/Air-T*) and homozygous *Air-T* mice showed a 15% reduction in birth weight as compared with wild-type littermates (data not shown). A similar phenotype caused by biallelic expression of *Igf2r* is seen in mice with a paternally inherited deletion of the 3.7-kb ICE; these mice do not express *Air* owing to
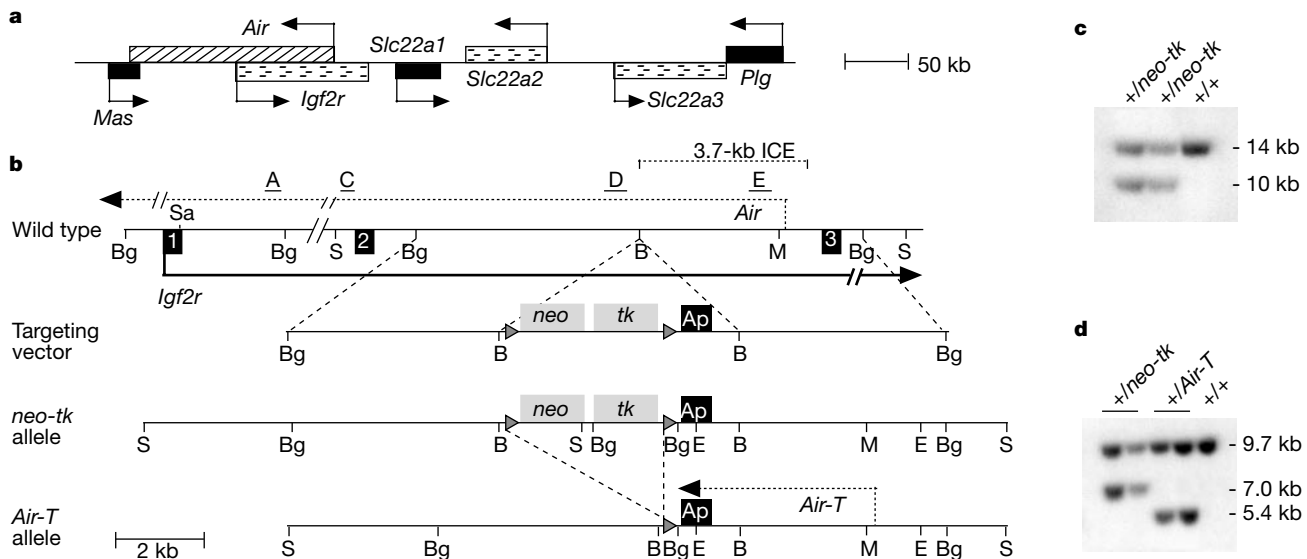

Figure 1 Generation of the *Air-T* allele. **a**, Map spanning the 400-kb imprinted cluster. Indicated are paternally expressed *Air* (hatched box), maternally expressed *Igf2r*, *Slc22a2* and *Slc22a3* (dotted boxes) and non-imprinted genes (black boxes). **b**, Wild-type allele comprises *Igf2r* (black arrow), *Igf2r* exons 1–3 (black boxes) and *Air* (dashed arrow). The locus was targeted at a *Bam*HI site bordering the 3.7-kb ICE (dotted line) that lies 3.0-kb downstream from the *Air* transcription start. The targeting vector contains a 1.2-kb rabbit β-globin gene polyadenylation cassette (Ap), and PGK– neomycin (*neo*) and PGK–thymidine-kinase (*tk*) selection genes flanked by loxP sites (triangles). Selection genes were deleted from the *neo-tk* allele by transient Cre transfection to generate the *Air-T* allele. Probes A, C, D and E are indicated. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; M, *Mlu*I; Sa, *Sal*I; S, *Sca*I. **c**, Targeted *neo-tk* clones were identified by a 10-kb *Sca*I fragment with probe C. Targeting was paternal-specific in 18 out of 18 ES clones (Supplementary Information). **d**, The *Air-T* allele was identified by a 5.4-kb *Bgl*II fragment with probe D.

deletion of the *Air* promoter[4].

We also analysed the *Air-T* allele in reciprocal, double hetero-zygous crosses with mice containing a 3-cM deletion that spanned this imprinted cluster (T^{hp}). In a wild-type background, maternal inheritance of the T^{hp} allele ($T^{hp}/+$) is lethal between 15.5 and 17.5 days postcoitum (d.p.c.), owing to the absence of *Igf2r*[4,13]. We found, however, that offspring with the T^{hp}/*Air-T* genotype were normal sized, viable and fertile, indicating that the maternal-specific T^{hp} phenotype had been rescued by the paternal *Air-T* allele (data not shown), as shown previously in crosses with a paternal 3.7-kb ICE deletion[4,5]. Offspring with the reciprocal genotype (*Air-T*/T^{hp}) showed similar size, viability and fertility (data not shown) to a $+/T^{hp}$ genotype.

We tested that the *Air* RNA expressed from the *Air-T* allele was truncated by using an RNase protection assay (RPA) with probes positioned across the 108 kb that is spanned by the endogenous *Air* RNA[6] (Fig. 2a). Probes downstream from the inserted polyadenyla-tion signal detected *Air* RNA from the paternal wild-type allele but not from the paternal *Air-T* allele, indicating the absence of stable *Air* RNA downstream from the polyadenylation cassette (Fig. 2b). We tested for polyadenylation at the inserted cassette by polymerase chain reaction with reverse transcription (RT-PCR) using a poly(dT) reverse transcription primer plus the PX6R gene-specific primer (Fig. 2a). Unexpectedly, RT-PCR generated a single 314-base-pair (bp) *Air-T*-specific fragment from a spliced, polyadeny-lated RNA that had used a splice donor located 53-bp downstream from the principal *Air* transcription start and a splice acceptor from the inserted exon 3 of the β-globin gene (Supplementary Infor-mation). The +53-bp splice donor has been identified in a transgene study but is not normally used in the wild-type locus; however, use

of this splice donor does not correlate with the loss of imprinting of *Igf2r*/*Air* in transgenes[6,14].

We investigated the proportion of spliced/unspliced *Air-T* RNA by RPA with probes positioned between the *Air* promoter and the inserted polyadenylation cassette (Fig. 2c). Both probe G and probe J showed that 20% unspliced *Air* RNA was present from the paternal *Air-T* allele as compared with the paternal wild-type allele (Fig. 2c). Probe K (comprising the RT-PCR fragment) detected both spliced and unspliced RNAs, and confirmed that these were present at 80% and 20%, respectively. Probe L spans the inserted polyadenylation signal and extends 404-bp downstream from the ATTAAA signal; this probe detected the same 173-bp protected fragment as found in the control LAI-313 transgene[14], showing that the ATTAAA signal is used, but not larger protected fragments (Fig. 2c). Thus, Fig. 2 shows that although the *Air-T* allele generates spliced and unspliced *Air* RNAs, these are both truncated 4.2-kb downstream from the *Air* promoter and stable RNA is not detected downstream from the polyadenylation cassette.

We tested the function of the 3.7-kb ICE in the *Air-T* allele by analysing the methylation state, imprinted expression and the expression level of the *Air* promoter in *Air-T* mice as compared with wild-type littermates. Methyl-sensitive enzymes identified methylation specific to the maternal allele on the *Air-T* promoter as found on the wild-type allele (Fig. 3a). Analysis by RPA showed imprinted, paternal-specific expression of the *Air-T* RNA as found on the wild-type allele, and also showed that the *Air-T* allele generates similar levels of stable *Air* RNA as compared with the wild-type allele (Fig. 3b). Although it is possible that insertion of the polyadenylation cassette disturbed an as yet unidentified imprinting element or altered transcription/stability of the *Air-T* RNA, the
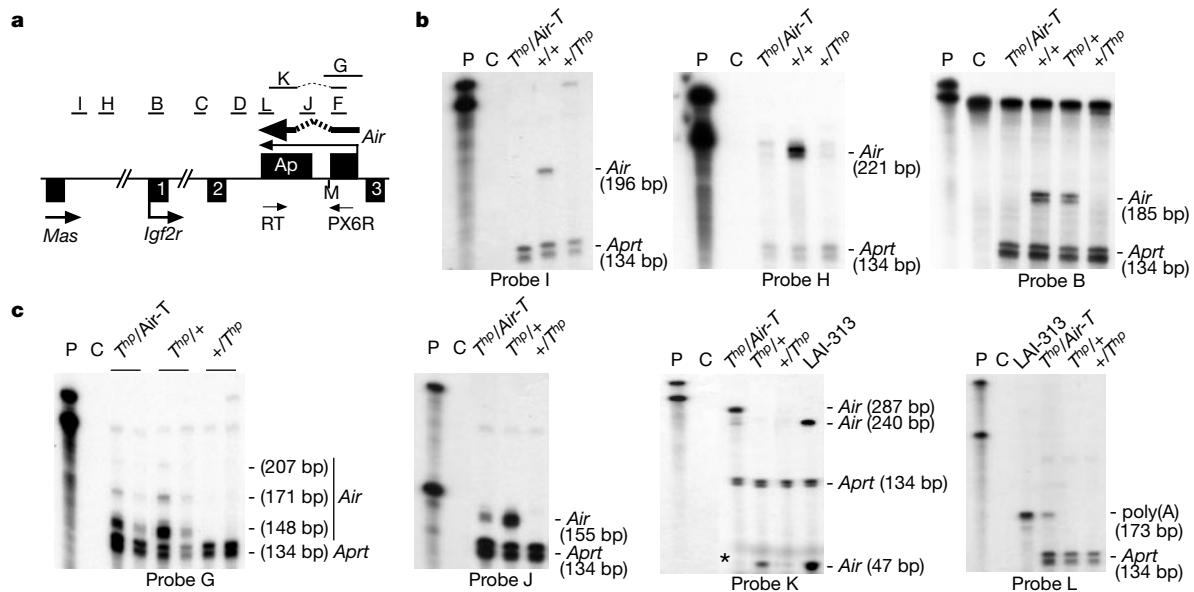

Figure 2 The *Air-T* allele truncates the Air RNA. **a**, Position of RNA probes (B−D, F−J, K (intron spanning), L), and RT and PX6R oligonucleotides used in RT-PCR, relative to those of *Mas*, *Igf2r* (exons 1−3) and *Air* on the *Air-T* allele. Unspliced *Air-T*, thin arrow; spliced *Air-T*, dashed arrow; *Air-T* exon 1, black box upstream from the *Mlu*I (M) site; polyadenylation cassette, Ap. **b**, RPA with probes B (on 11.5-d.p.c. embryo RNA), H and I (on adult heart RNA) indicates the absence of *Air* at, respectively, 28, 41 and 95 kb downstream from Ap. Identical results were obtained with probes C and D (Supplementary Information). **c**, RPA with probes G and J−L on 11.5-d.p.c. embryo RNA shows expression of truncated spliced and unspliced *Air-T*. Probe G (which overlaps a +53-bp splice donor and multiple *Air* transcription start sites) protects 207, 171 and 148 bp of unspliced *Air* RNA from the *Air-T* paternal allele. However, the signal quantified by phosphorimager was reduced by 80% compared with that of the wild-type paternal allele (note the relative loading of *Air-T* and $T^{hp}/+$ and that spliced fragments

protected by probe G are not visible). The signal reduction for unspliced *Air-T* is confirmed by probe J (which is located in the intron of the spliced *Air-T* RNA). Probe K is an RT-PCR fragment generated with oligonucleotides RT and PX6R (Supplementary Information) and protects 287 bp of spliced and 240 bp plus 47 bp of unspliced *Air-T* RNA (the 240-bp fragment is specific for the Ap cassette; asterisk indicates a faint 47-bp *Air* fragment). Probe K shows a ratio of 80% spliced and 20% unspliced *Air-T* RNA. The multicopy transgene LAI-313 contains the identical polyadenylation cassette[14], and more clearly protects the 240- and 47-bp unspliced *Air* fragments. Probe L spans the AATAAA polyadenylation signal and protects 173 bp of T^{hp}/*Air-T* and LAI-313. The absence of larger, protected probe-L fragments confirms that both the spliced and unspliced *Air-T* RNAs are truncated at the polyadenylation signal. In **b** and **c**, parental alleles are denoted maternal/paternal, the loading control is *Aprt* exon 3, input probes are indicated by P, and the tRNA control by C.

observation that the *Air-T* promoter was expressed and imprinted in a similar manner to that of the *Air* wild-type promoter indicates that function of the 3.7-kb ICE was not disturbed.

We examined the effect of the truncated *Air-T* RNA on paternal silencing of the imprinted cluster. Of the three genes in this cluster, only *Igf2r* becomes methylated when silenced on the wild-type paternal allele[5]. Methylation of *Igf2r* is completely absent from the paternal *Air-T* allele in contrast to the wild-type allele (Fig. 4a). We used RNA blots to assess imprinted expression and show (Fig. 4b, c) that all three genes, *Igf2r*, *Slc22a2* and *Slc22a3*, have a complete loss of silencing on the paternal *Air-T* allele. Maternal expression of all three genes from the *Air-T* allele was at the same level as maternal expression from the wild-type allele, indicating that the targeting event did not alter their expression.

The *Air-T* allele truncated the *Air* RNA to 4% of its length yet maintained the imprinted status and expression level of the wild-type paternal *Air* promoter (with the caveats noted above). This truncation caused a complete loss of silencing of the *Igf2r/Slc22a2/Slc22a3* cluster on the paternal chromosome. This shows that the *Air* non-coding RNA is required for *cis* repression of flanking protein-coding genes. The identification of a role for the *Air* non-coding RNA in *cis* repression contrasts with the previous observation that DNA insulator and silencer elements regulate imprinting of the *Ins2/Igf2/H19* cluster[10-12]. Our results thus identify a new RNA-dependent mechanism in genomic imprinting.

The bidirectional action of the *Air* RNA shown by our data is unexpected because the wild-type *Air* RNA overlaps only one gene.
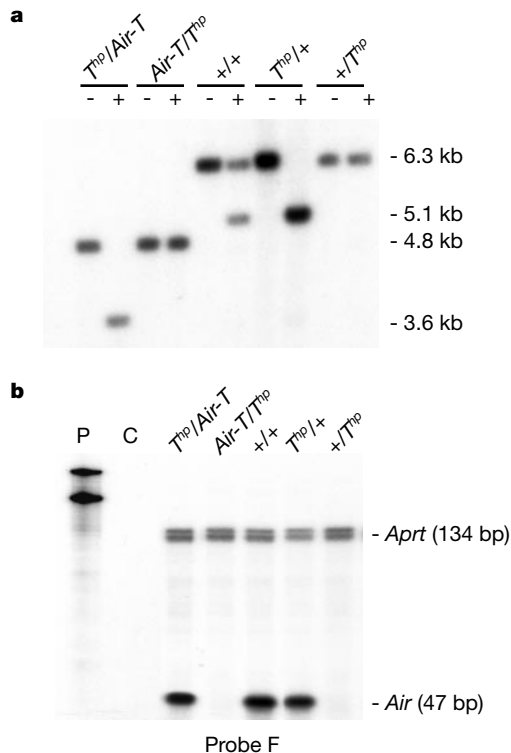
Repression, at least of *Slc22a2* and *Slc22a3*, does not require transcriptional overlap, yet overlap with non-coding RNAs has been found at five independent imprinted loci[3,15-18]. It is possible that the transcriptional overlap by non-coding RNAs may be involved if silencing of the non-overlapped genes were secondary to silencing of the overlapped gene. In this two-step model, the antisense non-coding RNA might repress the overlapped promoter (for example, by promoter occlusion or a form of *cis*-acting RNA interference; note that expression–competition[3] is excluded by our findings) and induce a silent chromatin state that could spread bidirectionally in a limited manner and silence flanking genes.

Alternatively, if transcriptional overlap is not involved in *cis* repression, then the non-coding RNAs might function to recruit repressor proteins to the gene cluster. This latter model has parallels with X inactivation in female mammals, in which expression of the non-coding *Xist* RNA inactivates one X chromosomes in *cis*[19]. The *Xist* RNA has features that have not been characterized as yet or that might be absent from imprinted non-coding RNAs, such as the ability to coat and spread along the length of the repressed chromosome[20-23]. Although further testing is clearly necessary to determine whether there are mechanistic similarities at the molecular level, it is notable that X inactivation has been suggested to have evolved from a localized form of imprinting that initially
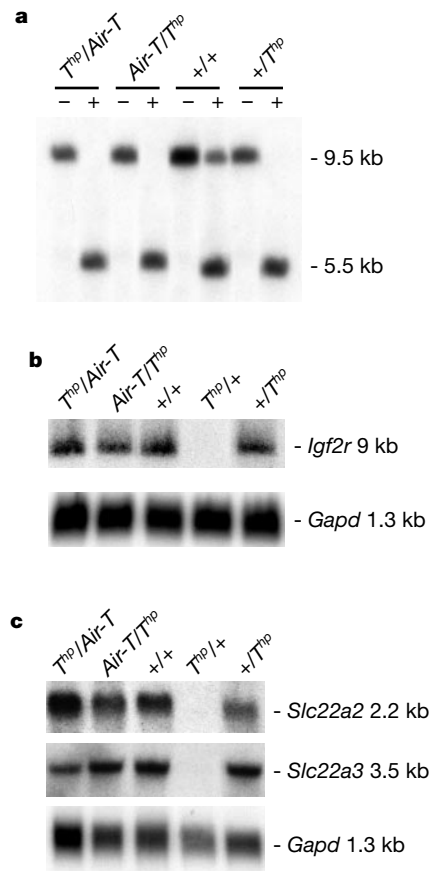

Figure 3 The *Air-T* allele is imprinted. **a**, The *Air-T* allele retains a methylation imprint on the *Air* promoter. DNA from 11.5-d.p.c. embryos cut with *Eco*RI (-) or *Eco*RI/*Mlu*I (+) was analysed with probe E. The fragments correspond to wild type methylated, 6.3 kb; wild type unmethylated, 5.1 kb; *Air-T* methylated, 4.8 kb; and *Air-T* unmethylated, 3.6 kb. Identical results were obtained with adult tail and spleen, and *Sfu*I (Supplementary Information). **b**, The *Air-T* allele maintains imprinted *Air* expression. RPA was carried out on 11.5-d.p.c. embryo RNA with probe F, which detects spliced and unspliced *Air*. The paternal *Air-T* and wild-type alleles show equal *Air* expression, whereas the maternal alleles repress *Air*. Identical results were obtained with heart RNA and probes G, J and K (Supplementary Information). P, input probes; C, tRNA control.

Figure 4 Loss of paternal silencing on the *Air-T* allele. **a**, The *Igf2r* promoter on the *Air-T* allele lacks paternal methylation. Tail DNA was cut with *Bgl*II (-) or *Bgl*I/*Sal*I (+) and analysed with probe A. Methylated fragment, 9.5 kb; unmethylated fragment, 5.5 kb. Identical results were obtained with adult spleen DNA and either *Not*I or *Sma*I (Supplementary Information). **b**, Loss of paternal *Igf2r* repression on the *Air-T* allele. RNA from 11.5-d.p.c. embryos was analysed with a probe covering exons 3–6 from *Igf2r*. Identical results were obtained with adult heart RNA (Supplementary Information). **c**, Loss of paternal *Slc22a2* and *Slc22a3* repression on the *Air-T* allele. RNA from 11.5-d.p.c. placenta was analysed with cDNA probes for *Slc22a2* and *Slc22a3*. In **b** and **c**, *Gapd* is a control for RNA loading.

affected only a small region of the X chromosome that contains a dosage-sensitive gene[24,25]. □

Methods

Generation of the *Air-T* allele

The targeting vector was constructed from a 9.6-kb *Bgl*II fragment (bp 117,878–127,575; AJ249895). A selection cassette containing *neomycin* and the thymidine kinase gene, each driven by a PGK promoter flanked by loxP sites plus a 1.2-kb fragment from the rabbit β-globin gene (bp 31,392–32,590; M18818) containing part of exon 2, complete intron 3 and complete exon 3 with the polyadenylation signal in the correct orientation for the *Air* promoter, was inserted at the *Bam*HI site in this fragment. The selection cassette was deleted by electroporation of a plasmid encoding Cre recombinase[26] and transient puromycin selection. We generated chimaeric mice by injecting paternally targeted *Air-T* embryonic stem (ES) cells into C57/Bl6 blastocysts[27]. Mice carrying the *Air-T* allele were maintained on an FVB/N background.

Methylation and expression analyses

Digestion of methyl-sensitive enzymes was monitored by hybridization to mitochondrial DNA. We carried out RPA using an RPAIII kit (Ambion). Signals were quantified with a Phosphorimager (Fujix). For RT-PCR, 5 µg heart RNA was reverse transcribed with Superscript (Gibco) using the linker-poly(dT) primer RT, 5′-CTGGGAAACAGCTATGACCATGATCGATTTTTTTTTTTTTTTTTTTN-3′, and amplified with PX6R, 5′-GAAGCACAGCACCGCCAGTTAC-3′, for 30 cycles (94 °C, 30 s; 59 °C, 30 s; 72 °C, 120 s).

Probes for DNA analyses

We used the following probes: probe A, a 325-bp PCR fragment (bp 102,813–103,137; AJ249895); probe C, a 0.9-kb *Eco*RI/*Hin*CII fragment from EST AA592338 (ref. 6) collinear with genomic DNA; probe D, (bp 121,834–122,862; AJ249895); and probe E (bp 124,992–126,086; AJ249895).

Probes for RNA analyses

Probe B, described as probe GFPAIR[14], is 322 bp and protects 185 bp of *Air* RNA at the *Igf2r* promoter. Probes C and D are the same as the DNA analyses probes. Probe F is 174 bp and protects 47 bp (bp 126,181–126,227; AJ249895) immediately downstream from the principal *Air* transcription start site[6]. Probe G (bp 126,086–126,293; AJ249895), described as probe MlMs1 (refs 6,14), detects unspliced (207, 171 and 148 bp) *Air* RNA fragments. This relatively large probe did not produce a clear signal for the spliced *Air-T* RNA (predicted fragments of 112, 76 and 53 bp), which should have been recognized (see probe K below). Probe H, described as probe RPA1 (ref. 6), protects 221 bp of *Air* RNA (bp 85,250–85,029; AJ249895). Probe I, described as probe MS1B8 (ref. 6) protects 196 bp. Probe J is 173 bp and protects 155 bp of *Air* RNA (bp 123,233-123,387; AJ249895). Probe K is 364 bp, contains the RT-PCR fragment made with probes RT and PX6R (Supplementary Information) and protects 287 bp of spliced and 240 and 47 bp of unspliced polyadenylated RNA. Probe L spans 558 bp (bp 32,032–32,590; M18818) of the β-globin gene (including the polyadenylation signal) and protects 173 bp of polyadenylated RNA (bp 32,032–32,204; M18818). Probe L extends 385 bp downstream from the polyadenylation signal and the absence of protected fragments longer than 173 bp indicates use of the polyadenylation signal. The *Aprt* template is a 252-bp *Xho*I/*Xba*I fragment (bp 2,165–2,417; M11310) and protects *Aprt* exon 3 (134 bp). For RNA blots, we used the following probes: for *Igf2r*, complementary DNA exons 3–6; for *Slc22a2*, bp 989–1,605 (AJ006036); for *Slc22a3* bp 1–2,766 (AF078750); for *Gapd*, complete cDNA.

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Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPARα

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Repression of gene transcription by nuclear receptors is mediated by interactions with co-repressor proteins such as SMRT and N-CoR[1,2], which in turn recruit histone deacetylases to the chromatin[3–5]. Aberrant interactions between nuclear receptors and co-repressors contribute towards acute promyelocytic leukaemia and thyroid hormone resistance syndrome[6–8]. The binding of co-repressors to nuclear receptors occurs in the unliganded state, and can be stabilized by antagonists[9]. Here we report the crystal structure of a ternary complex containing the peroxisome proliferator-activated receptor-α ligand-binding domain bound to the antagonist GW6471 and a SMRT co-repressor motif. In this structure, the co-repressor motif adopts a three-turn α-helix that prevents the carboxy-terminal activation helix (AF-2) of the receptor from assuming the active conformation. Binding of